Investigating the Importance of Transcription Factors on Proliferative Pathways in Cancer

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Abstract

Transcription factors (TFs) are the primary mechanism that cells use to respond to external stimuli. These TFs allow for inducible regulation of gene expression. For this reason, TFs can act as a proto-oncogenes, tumor suppressors, or secondary messengers and aid many hallmarks of cancer. Understanding how TFs interact in cancer-causing pathways could lead to a much better understanding of the disease. In both the Guttridge and Miles laboratories, the impact of transcription factors in cancer-causing pathways, NF-κB in the former and RB in the latter, was investigated. Elucidating the function of lesser-known molecules in these pathways could profoundly impact how we understand the development of several different types of cancer.

The NF-κB pathway plays an important role in controlling the immune response. It is also constitutively activated in leukemia/lymphoma, AML, breast, colon, pancreas, liver, head and neck cancers and is activated by many oncogenes (Ras, Her2/neu, BCR-ABL, HTLV-1/TAX and et al). In transformed cells, NF-κB is thought to suppress the immune system and allow for tumor development. 35 possible candidate genes were tested for NF-κB-regulation with qRT-PCR. 12 of these genes showed differential gene expression between wild-type and mutant NF-κB cell lines, showing that they could be involved in immune suppression around cancer cells.

The RB pathway, controlled by RB1, RBL1, and RBL2 genes, and the pocket proteins (pRB, p107, and p130) that these genes encode are critical for regulating E2Fs that promote cell proliferation and apoptosis. pRB is important tumor suppressor that is inactivated in almost all human cancers and E2F functions are also commonly mis-regulated in cancer. Retinoblastoma (RB), a rare tumor of the retina caused by the homozygous loss of RB1, is thought to originate in rod and cone retinal pigment epithelial (RPE) cells.

Retinoblastoma susceptibility gene (RB1), which encodes pRB, was first tumor suppressor gene ever identified. However, the functions RBL1 and RBL2, which encode p107 and p130 respectively, are still unknown despite encoding similarly structured proteins and having similar importance to the RB pathway as RB1. CRISPR-Cas9 gene editing was used to create two knockout (KO) RPE cell-lines for each RBL1 and RBL2. Western Blotting confirmed that both RBL2 cell lines were homozygous CRISPR-KOs while the RBL1 cell lines were a homozygous CRISPR-KO and a heterozygous CRISPR-KO.

Introduction

Cells must respond to external stimuli to develop and differentiate. The primary way that cells can modulate their gene expression profile to respond to these effects is via transcription factors (TF). These molecules bind to specific response elements (RE) in DNA promoter regions to either recruit RNA polymerases (Pol), including Pol II, or to bind to REs irreversibly (Latchman, 1997). As a result, TF binding to *cis*-regulatory DNA sequences can turn genes "on" or "off." (Phillips et al, 2008). This inducible regulation of gene expression is a critical aspect of the ability of multicellular organisms to adapt to environmental, mechanical, chemical, and microbiological stresses (Hayden, M. S. et al, 2008).

Transcription factors have a central role in cancer biology as they are capable of acting as a proto-oncogenes, tumor suppressors, or secondary messengers. Cancer is "the uncontrolled overgrowth of a particular cell type, initiated with an unwanted mutation in one or more genes, followed by an increasing accumulation of defects in hundreds if not thousands of genes, as a function of time, leading to invasive malignancy" (Bartek and Lukas, 2001). TFs play major supporting roles in cancer development, aiding many hallmarks of cancer: cell survival, cell growth, cell differentiation, proliferation, immune suppression, inflammation, and angiogenesis (Courtois et al, 2006) (Hamik et al, 2006). It is therefore unsurprising that TFs account for 20% of all oncogenes that have been discovered (Lambert et al, 2018). Given the overall importance of transcription factors to cancer development, discovering how TFs interact in cancer-causing pathways could be critical to understanding the disease more comprehensively.

NF-κB

The Nuclear Factor kappa-light-chain-enhancer of activated B cell (NF-κB) family of transcription factors is likely the most intensely researched TF family. Around 10% of all articles on PubMed about TFs are on NF-κB (Chaturvedi, M.M. et al, 2011), a rational development since the NF-κB pathway is a major mechanism used by the body to respond to stimuli.

NF-κB regulates many functions including cell proliferation, cell survival, the cellular stress response, and inflammation (Courtois et al, 2006). In particular, this pathway plays an important role in controlling the immune response. NF-κB initially regulates the expression of genes during embryonic development, shaping the development of the mammary gland, bones, skin, and central nervous system (Hayden, M.S. et al, 2004). NF-κB regulates cytokines, growth factors, and effector enzymes in response to aspects of both innate and adaptive immunity (Hayden, M.S. et al, 2004). In the context of innate immunity, Toll-like receptor (TLR) signaling spurred on by a wide variety of pathogens can lead to activation of NF-κB and production of inflammatory proteins (Bonizzi et al, 2004). As an important aspect of adaptive immunity, antigen receptor binding leads to a signaling cascade that activates NF-κB activation and causes the release of cytokines that drive B-cell differentiation (Bonizzi et al, 2004).

The NF-κB pathway [Figure 1] is activated via an IκB kinase (IKK) complex consisting of three subunits: inhibitor of nuclear factor kappa-B kinase subunit alpha (IκKα), inhibitor of nuclear factor kappa-B kinase subunit beta (IκKβ), and inhibitor of nuclear factor kappa-B kinase subunit gamma (IκKγ). This IKK complex phosphorylates IκBα, which causes subsequent

ubiquitination and degradation of the IκBα protein, thus releasing the p50/p65 dimer into the nucleus to activate transcription (Wang, J et al, 2009).

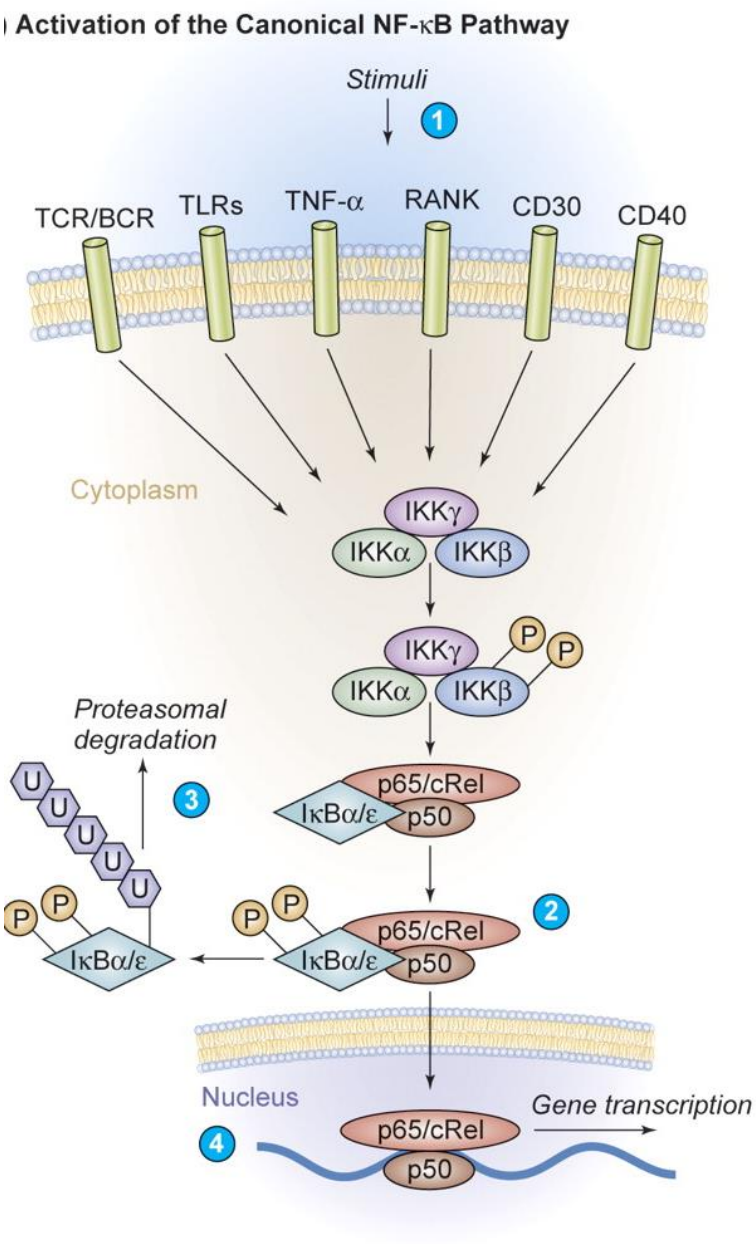

Figure 1: The Classical NF-κB pathway (Jost P. J. and Ruland J, 2007)

Given the importance of NF-κB in many functions of healthy cells, it is unsurprising that the mis-regulation of the pathway can have damaging consequences. Mutations and/or epigenetic

alterations of the pathway have been implicated in many human and animal diseases, especially those associated with chronic inflammation, immunodeficiency or cancer (Courtois et al, 2006). NF-κB has anti-apoptotic activity, which usually aids the growth of the progenitors of immune cells, and can be used to enable replicative immortality of cancer cells (Hanson et al, 2004). Additionally, the inflammation response that NF-κB can induce could be used to promote tumor growth. Lastly, NF-κB normally functions as a tumor suppressor but can switch to tumor promoter during Ras-mediated transformation. This allows for the suppression of the immune system around tumor cells (Wang, D. J. et al, 2014).

RB, RBL 1, and RBL 2

The retinoblastoma susceptibility gene (RB1) was the first tumor suppressor gene to be identified (Friend et al, 1986). The protein it encodes, pRB, serves as a negative regulator of cell proliferation (van den Heuvel et al, 2008). As a negative regulator, pRB has a role in temporary and permanent cell cycle arrest, genomic stability, apoptosis and differentiation (Burkhart et al, 2008). pRB is an important tumor suppressor that is inactivated, directly or indirectly, in almost all human cancers (Burkhart et al, 2008).

The RB1 gene is part of a larger gene family that includes RB Transcriptional Corepressor Like 1 (RBL 1) and RB Transcriptional Corepressor Like 2 (RBL 2). These three genes encode structurally related “pocket proteins” pRb, p107, and p130, respectively (Di Fiore et al, 2013). pRB inhibits the E2 Promoter Binding Factor (E2F) proteins, transcription factors that promote the transcription of mRNAs involved in both cell proliferation and apoptosis (Miles et al, 2014).

The E2F family of transcription factors activate genes that are necessary for the G1/S-phase transition (van den Heuvel et al 2008). RB family proteins bind to E2Fs in a temporal fashion, inhibiting E2F transcriptional activities in the early G1 phase of the cell cycle. In late G1 to S phase, cyclin-dependent kinases (CDKs) phosphorylate the RB family proteins, causing them to dissociate and freeing E2Fs to activate E2F-dependent transcription (Burkhart et al, 2008). Like pRB, E2F transcription factor functions are commonly mis-regulated in cancer (Ji, J.Y. et al, 2012).

The activities of E2F1-5 are negatively regulated by their specific interaction with pocket proteins (pRB, p107, p130) (Dimova et al, 2003). The E2F family is broken up into two subgroups: activator E2Fs and repressor E2Fs. Activator E2Fs are expressed at specific times in the cell cycle and promote cell cycle progression, while repressor E2Fs are expressed during the entirety the cell cycle but are specifically required for cell cycle exit and differentiation (Wikenheiser-Brokamp K.A, 2006).

pRB is structurally related to p107 (encoded by RBL 1) and p130 (encoded by RBL 2) but the latter two pocket proteins are much more structurally related to one another than to pRB (Classon and Dyson, 2001). Additionally, p107 and p130 only associate with the repressor E2Fs, namely E2F4 and E2F5, while pRB binds primarily to activator E2Fs, like E2F1, E2F2, and E2F3a, as well as one repressor, E2F3b [Figure 2] (Cobrinik, 2005).

There are a few differences in expression among pRB, p130, and p107 in proliferating, nonproliferating and quiescent cells. pRB expression is seen in both proliferating and nonproliferating cells, p107 expression is most prominent in proliferating cells, and p130 expression is primarily seen in arrested cells (Classon and Dyson, 2001). However, these

differences don't necessarily rule out p130 or p107 as negative regulators of cellular proliferation.

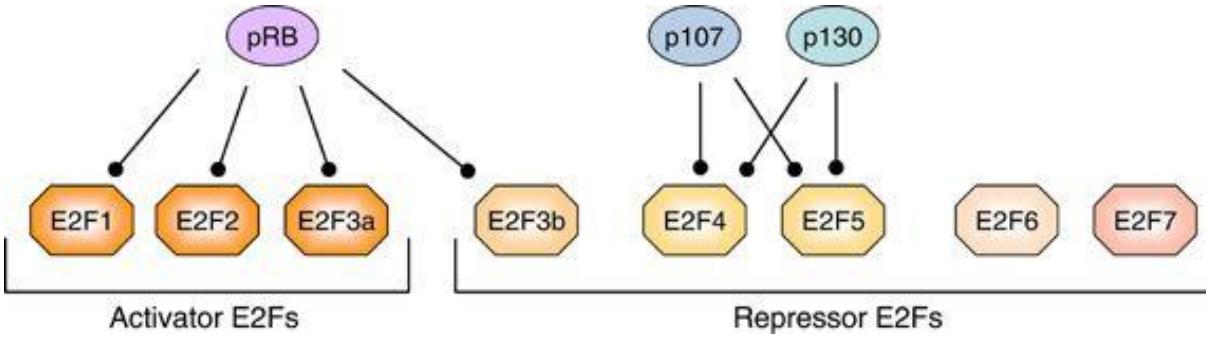

Figure 2: Interactions among the pocket proteins and members of the E2F family. pRB binds primarily to activators (E2F1, E2F2, E2F3a) as well as one repressor (E2F3b). p107 and p130 only bind to repressor (E2F4, E2F5) (Cobrinik, 2005).

Guttridge Laboratory

I led my own project that used quantitative/real-time reverse transcription polymerase chain reaction (qRT-PCR) to explore candidate target genes for a cancer-causing pathway, NF-κB. This project focused on measuring how NF-κB can function in cancer cells to counter immune elimination by the innate and adaptive immune systems. As a *bona fide* regulator of the immune response (Hayden, M.S. et al, 2004), NF-κB supports cancer development by promoting cell survival, cell growth, proliferation, metastasis, and epithelial to mesenchymal transition (EMT) (Chaturvedi, M.M. et al, 2011).

NF-κB is also constitutively activated in several cancers or cancer cell lines (leukemia/lymphoma, acute myeloid leukemia, breast, colon, pancreas, liver, head and neck cancers) and is activated by many oncogenes (Ras, Her2/neu, BCR-ABL, HTLV-1/TAX). The NF-κB family of proteins function as dimers from 5 subunits, with the p50/p65 heterodimer being expressed in almost all mammalian cells (Wang, J et al, 2009).

The p65 subunit of NF-κB acts as a tumor suppressor in normal cells by sustaining senescence following DNA damage (Wang, J et al, 2009). However, recent findings from the Guttridge Lab reveal that, following immortalization, p65 switches to an oncogene by counteracting the surveillance properties of immune cells (Wang, D. J. et al, 2014). This would indicate that NF-κB can also function in cancer cells to counter anti-tumor immune cells.

NF-κB exerts this effect by protecting transformed cells against macrophage-derived proapoptotic factors, tumor necrosis factor, and nitric oxide [Figure 3] (Wang, D. J. et al, 2014). Evidence for protection against macrophages is shown by a greater survival rate amongst cancer cells in wild-type cell lines relative to NF-κB null lines [Figure 4] (Wang, D. J. et al, 2014). Additionally, in severe combined immune deficiency (SCID) mice, tumor growth is much faster in wild type Ras cells than *p65*$^{-/-}$*Ras* cells [Figure 5] (Wang, D. J. et al, 2014).

Previous experiments conducted by researchers in the Guttridge Lab, comparing *p65*$^{+/+}$*Ras* and *p65*$^{-/-}$*Ras* Mouse Embryonic Fibroblasts (MEFs) by RNA sequencing, identified 1,946 genes as being downregulated in *p65*$^{-/-}$*Ras* cells (Ratnam et al, 2017). Once grouped based on their biological function, 51 were classified as genes that participate in cellular movement, secretion, enzyme production, and gene expression. The goal of this project was to discover all remaining unpublished target genes of NF-κB regulation to get an extensive view of how the NF-κB pathway allows cancer cells to evade the immune system.

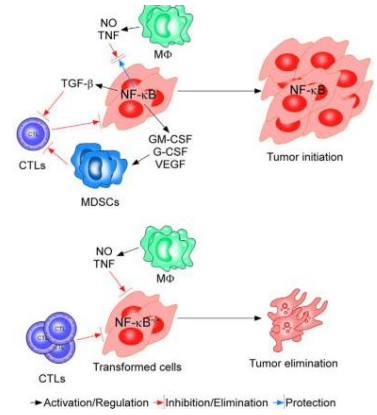

Figure 3: Model for NF-κB protection of tumor cells against macrophages (Wang, D. J. et al, 2014)

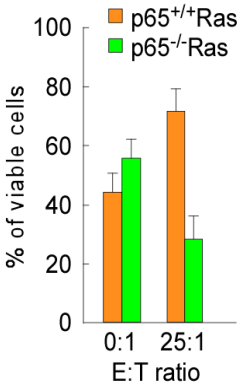

Figure 4: NF-κB increases survival in higher effector to target ratios. Data is from 3 experiments ($p<0.001$). (Wang, D. J. et al, 2014)

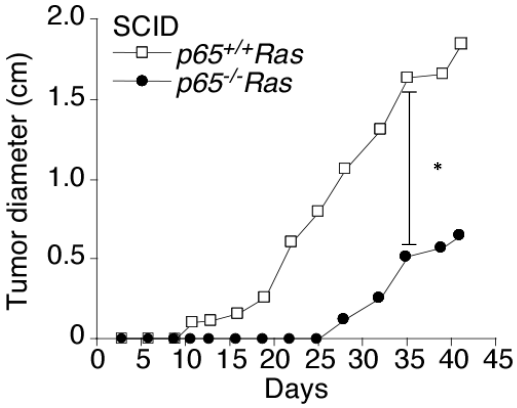

Figure 5: Tumor growth among *p65$^{+/+}$Ras* and *p65$^{-/-}$* SCID mice. Data is from 16 mice injected ($p = 0.004$). (Wang, D. J. et al, 2014)

Miles Laboratory

My project is focused on the RB pathway, attempting to elucidate the roles of the RBL 1 and RBL 2 genes. Retinoblastoma (RB), a rare tumor of the retina caused by the homozygous loss of RB1, is thought to originate in rod and cone retinal pigment epithelial (RPE) cells, (Xu X. L. et al, 2009). The subsequent loss of the RB1 protein, pRB, results in loss of E2F regulation, chromatin changes and developmental defects leading to tumor development (Elchuri et al, 2018).

With RB1 being a major regulator of multiple processes in a cell, pRB loss was expected to majorly change the cellular landscape – specifically, result in increased cellular proliferation. However, pRB-depletion in model organisms has only a small effect on accelerating growth rates. This was shown to be partly because of compensatory mechanisms by p107 and p130 (Xu X. L. et al, 2014).

When either of these lesser-known pocket proteins are deficient in mouse models, there are massive phenotypic impacts. p107 null mutants (*p107*$^{-/-}$) had increased neural progenitor cell proliferation and apoptosis, subtle thickening of long bones, impaired growth, and a myeloproliferative disorder while *p130*$^{-/-}$ mutants occasionally suffered lethality with aberrant neural, muscle and heart development (Cobrinik et al, 1996). *p107*$^{-/-}$, *p130*$^{-/-}$ double mutants all suffered lethality at birth and had aberrant endochondral bone development, increased epidermal proliferation and aberrant differentiation, and delayed development of hair, whiskers and teeth (Cobrinik et al, 1996). Lastly, both *pRb*$^{-/-}$, *p107*$^{-/-}$ and *pRb*$^{-/-}$, *p130*$^{-/-}$ double mutants developed retinoblastoma and had enhanced apoptosis in the central nervous system (CNS) and liver (Dannenberg J. H. et al, 2004).

The compensatory mechanisms shown in the presence of pRb-depletions, phenotypic changes seen in the absences of any pocket protein, and similarities in function among pRB, p107, and p130 still perpetuate the idea that both RBL 1 and RBL 2 function as tumor suppressors in the RB pathway. Despite the important role played by these proteins in the RB tumor suppressor pathway, the biological role of these proteins in normal human cell lines have not yet been identified.

Methods

Guttridge Laboratory

Target gene search

My project in the Guttridge Lab investigated the role of the 51 genes shown to participate in cellular movement, secretion, enzyme production, and gene expression pathways. These genes were further subjected to NF-κB regulated gene data base search (Gilmore et al, 2012). 16 genes were already published as NF-κB regulated genes, therefore these genes were eliminated from our study, leaving 35 as possible NF-κB target genes that were not previously reported.

Primer design

The mRNA sequences of each of the target genes in *Mus Musculus* were found using the National Center for Biotechnology Information (NCBI) gene database. Primers for each gene were designed with a web-based primer program from the mRNA sequence (primer 3.1). The most viable forward and reverse primers for each gene were checked for dimers based on complementary sequences within the mRNA sequence through Amplify. Primers for each gene target were ordered as 20 base-pair oligos from IDTDNA. Primers from IDTDNA were centrifuged then reconstituted to 100 uM with 100-200 uL ddH_2O. These primers were further diluted 1:10 with 1-2 mL ddH_2O. Lastly, the mixtures were put into primer plates and diluted on a 1:1 basis with ddH_2O [Figure 6].

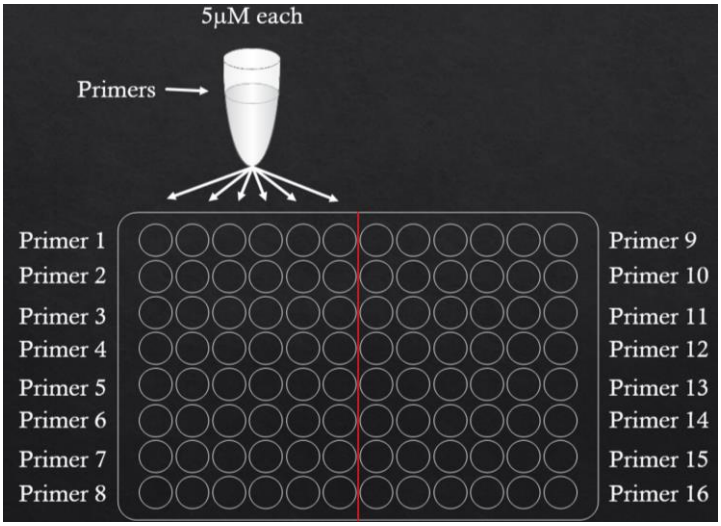

Figure 6: Primer dilution plate; 5 uM (1 uL) of each primer was placed in six wells to be diluted then transferred onto a PCR plate

RNA Preparation

RNA was then prepared from two independent lines of *p65*$^{+/+}$*Ras* and *p65*$^{-/-}$*Ras* MEFs. Adherent cells were cultured to 70 – 80% confluence and washed twice with ice cold Phosphate buffered saline (PBS). 1-3 mL Invitrogen TRIzol was added to each dish of cultured cells and the cells were scraped to one side of the dish. The suspensions were pipetted up and down several times to lyse the cells.

These mixtures were incubated at room temperature for 5 minutes. 200 uL chloroform was then added to each 1 mL cell lysate. The mixtures were shaken vigorously for 15 seconds before incubation at room temperature for 2-3 minutes. These solutions were centrifuged at 14000 rpm for 15 minutes. The supernatants were transferred to new tubes and the same volumes of isopropanol were added before mixture and incubation at room temperature for 10 minutes. The mixtures were then centrifuged at 14000 rpm for 10 minutes.

The newest supernatants were poured off and 700 mL 75% ethanol was added before the tubes were inverted several times. The mixtures were then centrifuged at 14000 rpm for 10 minutes and air dried for 15 – 20 minutes. The pellets were dissolved into DEPC-ddH_2O and its OD was measured with a Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer to measure the Total RNA concentration. To confirm RNA purity and quality, 0.2-0.5 ug of each RNA was run on 1% TAE agarose gel (10 mL 1X TAE, 1 g agarose), which contained an RNA reference ladder with discrete ribosomal peaks of 28S, 18S and 5S, to be visualized on an Agilent 2100 Bioanalyzer.

cDNA Preparation

Half of this extracted RNA was further reverse transcribed into cDNA. 5 ug RNA was diluted with a mixture of ddH_2O, 10 uL of basic primer (0.1ug/ul) diluted with oligodT (0.5ug/uL), and 1 uL of PCR specific primer for each gene tested. This mixture was incubated at 70 ^oC for 10 minutes then room temperature for 5-10 minutes then on ice. It was mixed with 10 uL 5x 1st strand buffer, 5 uL 0.1M DTT, 10 uL of 10mM dNTP, 2 uL Rnase inhibitor, 5-10 uL ddH_2O (up to 48ul, dependent on initial concentration of RNA) and 2 uL M-MLV RTase. The solution was then gently vortexed and incubated in 37 ^oC water bath for 1 hour before another incubation at 70 ^oC for 10 minutes. It was put on ice for 2 minutes before being diluted with ddH_2O so there was 10 – 20 ng of cDNA per uL.

qRT-PCR analysis

$p65^{+/+}$Ras and *$p65^{-/-}$Ras* (MEF) cDNA as well as *$p65^{+/+}$Ras* and *$p65^{-/-}$Ras* RNA [negative control for measure of purity] were subjected to real time quantitative PCR analysis in duplicate with SYBR Green PCR kits to test for differential expression and primer quality. Each gene

sample was loaded into triplicate wells and mouse B-actin was used as the control. Every well contained 10 uL of 2X Roche SYBR Green PCR Master Mix, 6 uL ddH_2O, 2 uL of (upstream and downstream) primer, and either 2 uL of $p65^{+/+}$ cDNA, $p65^{-/-}$ cDNA, $p65^{+/+}$ RNA, or $p65^{-/-}$ RNA [Figure 7].

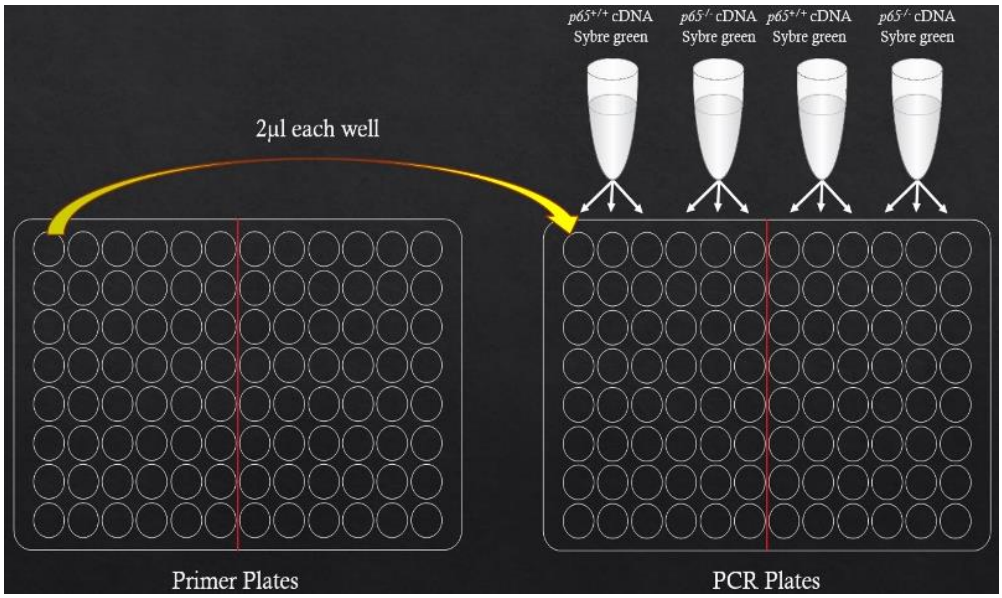

Figure 7: PCR plate; 2 uL from each well in the primer plate [Figure 6] was pipetted into the corresponding well of the PCR plate

This PCR plate was put into an Applied Biosystems StepOnePlus RT-PCR machine set at:

Step	Activation	Amplification		
	Hold	denature	annealing	elongation
Temperature	95°C	94°C	50-60°C	72°C
Time	10min	15 sec	30 sec	20-60 sec
Cycle	-	35-45		

A comparison test was performed after the first qRT-PCR round using ΔCT and normalizing with a housekeeping gene in the MEF *p65*$^{+/+}$ wells and *p65*$^{-/-}$ wells. The significance

point for all genes was set to having expression on the $p65^{-/-}$ cDNA wells be less than or equal to 70% of the expression on $p65^{+/+}$ cDNA wells.

A T-test was performed after the second qRT-PCR round. The intent was to use ΔCT and normalize with a housekeeping gene to compare the expression of each gene on three more wells of each $p65^{+/+}$*Ras* and $p65^{-/-}$*Ras* to see if any of the genes that these primers represent had differential expression between the wild-type and mutant strains. The significance point for all genes was set to having expression on the $p65^{-/-}$ cDNA wells be less than or equal to 70% of the expression on $p65^{+/+}$ cDNA wells.

Miles Laboratory

Guide RNA Design

My project in the Miles Lab involves the CRISPR-mediated knock out (KO) of RBL 1 and RBL 2 genes in a normal human RPE1 cell line. I designed guides with ChopChop program from MIT and used these to order properly designed guide RNAs for the two genes (RBL 1 and RBL 2) that I am attempting to knockout of cell lines. Once received, these guides were reconstituted to 100 uM in H_2O.

Gene KO	Forward Guide	Reverse Guide
RBL 1-1	CACCGTGGTGATCCGCTCAGAAGAG	AAACCTCTTCTGAGCGGATCACCAC
RBL 1-2	CACCGCGCTACAGTTCTCCTACCGC	AAACGCGGTAGGAGAACTGTAGCGC

RBL 2-1	CACCGCCGCCTCAACATGGAC GAGG	AAACCCTCGTCCATGTTGAGG CGGC
RBL 2-2	CACCGGGCGACTGGTCACCTC CCGA	AAACTCGGGAGGTGACCAGTC GCCC

Vector Preparation

Vector was then midi-prepped with a QIAGEN midiprep kit for use in cloning. 2 mL of LB media, 2 uL ampicillin, and cells were picked from colonies of each cell line mixed together and incubated on a shaker (250 rpm) at 37 ^{o}C overnight. 2 mL of every mixture was then added to 48 mL of LB media each and incubated on a shaker (250 rpm) at 37 ^{o}C overnight. These cultures were harvested by centrifuging at 6000 g for 15 minutes at 4 ^{o}C. The pellets were resuspended in 4 mL of P1 buffer. 4 mL of P2 buffer was added and the solution was mixed by inversion and incubated at RT for 5 minutes. 4 mL of P3 buffer was then added, followed by inversion and incubation on ice for 15 minutes. The mixtures were centrifuged at 20,000 g for 30 minutes at 4 ^{o}C.

QIAGEN-tips were equilibrated with 4 mL QBT buffer then supernatants added to tips, entered resin by gravity flow. The tips were washed with 2 rounds of 10 mL QC buffer. DNA from each tip was eluted with 5 mL QF buffer into clean vessels. The DNA was then precipitated by adding 3.5 mL isopropanol and mixing. The mixtures were centrifuged at 20,000 g for 30 minutes at 4 ^{o}C and the supernatants were decanted. The remaining DNA pellets were washed with 5 mL 70% ethanol and centrifuged at 20,000 g for 10 minutes and the supernatants were decanted. The final pellets were air-dried for 10 minutes then dissolved in 100 uL of endotoxin-free TE. The vector DNA was digested with restriction enzymes by mixing 5 ug of DNA, 2.5 uL

BsmB1, and 10 uL 10x NEB Buffer for each vector. The mixtures were incubated for 4 hours at 37ºC.

The DNA was then treated through gel purification to isolate and purify DNA fragments based on size. Loading buffer was added to each of the DNA samples. The agarose gel was placed into the electrophoresis unit which was filled with 1x TAE until the gel was covered. A molecular weight ladder was loaded into the first lane of the gel while the DNA samples were loaded into the additional wells of the gel. The gel was placed in a BIO-RAD Wide Mini-Sub Cell GT, which was attached to a BIO-RAD Power Pac and run at 120 V then ramped up to 150 V for 2 hours. DNA fragments were visualized with UV light.

The DNA band was excised from the agarose gel using a QIAGEN QIAEX II Gel Extraction Kit. 3 volumes of QX1 buffer were added to 1 volume of gel. QIAEX II was resuspended by vortexing for 30 s and 30 µl of QIAEX II was added. The solution was incubated at 50°C for 10 min and mixed by vortexing every 2 min to keep QIAEX II in suspension. The sample was then centrifuged for 30 seconds and supernatant was removed. The pellet was washed with 500 µl of Buffer QX1 and resuspended by vortexing. It was centrifuged for 30 seconds before all supernatant was removed. The pellet was then washed twice with 500 µl PE buffer. It was again resuspend by vortexing then centrifuged for 30 seconds before all supernatant was removed. This pellet was air-dried for 10–15 min, resuspended with 20 µl of 10 mM TrisCl, and mixed by vortexing. This solution was incubated at 50°C for 5 minutes before centrifuging for 30 seconds and removing supernatant.

Cloning

After the vector was prepared, the cloning process began. 100 uM (2.62 uL) of forward and reverse guides were diluted with 44.76 uL of ddH_2O for every gene. This mixture was run in a Thermocycler at 95 ^oC for 5 minutes then ramped down by 0.1 ^oC to 25 ^oC and allowed to sit until the next step. The annealed oligos were diluted 1:200 with ddH_2O and incubated at room temperature for 1 hour.

1 uL of the diluted oligo duplex, 5 uL 2x Electroligase buffer, 3.1 uL ddH_2O, 0.9 uL of BsmBI digested Blast plasmid, and 1 uL Electroligase were mixed for each gene. The mixture was inactivated at 65 ^oC for 15 minutes in water bath. 2 uL of each mixture was put into 50 uL of Stbl3 competent cells. The solutions put into 2 mm cuvettes and pulsed in a BIO-RAD MicroPulser at the Ec2 (2.5 kV/pulse) setting. The shocked solutions were put into 1 mL of SOC and incubated in a shaker for 2 hours at 37 ^oC. They were then spun down at 6000 g for 3 minutes and resuspended in 50 uL of SOC. Lastly, the solutions were plated on ampicillin plates and incubated at 37 ^oC overnight.

2 mL of LB media, 2 uL ampicillin, and cells picked from colonies of each cell line were mixed together and incubated on a shaker (250 rpm) at 37 ^oC overnight. 2 mL of every mixture was then added to 48 mL of LB media each and incubated on a shaker (250 rpm) at 37 ^oC overnight.

DNA Extraction-Miniprep

DNA was extracted from each of these cultures through a QIAGEN mini prep protocol. Pellets were harvested by centrifuging at 6000 g for 15 minutes at 4 ^oC then resuspended in 300

uL of P1 buffer. 300 uL of P2 buffer was added to each of the solutions, which were mixed by inversion and incubated at 24 ^{o}C for 5 minutes. Next, 300 uL of P3 buffer was added and the solutions were mixed by inversion then incubation on ice for 5 minutes. The mixtures were centrifuged at 14,000 g for 10 minutes at 4 ^{o}C.

In the mean-time, QIAGEN-tips were equilibrated with 1 mL QBT buffer. The supernatant of each solution was added to a tip and entered resin by gravity flow. The tips washed with 2 rounds of 2 mL QC buffer. DNA from each solution was eluted with 800 uL QF buffer into clean vessels. This DNA was precipitated by adding 560 uL isopropanol and mixing. The mixtures were centrifuged at 20,000 g for 30 minutes at 4 ^{o}C before their supernatants were decanted. The DNA pellets were washed with 1 mL 70% ethanol and centrifuged at 20,000 g for 10 minutes before their supernatants were decanted. The final pellets were air-dried for 10 minutes then dissolved in 20 uL of endotoxin-free TE.

Sanger sequencing with the help of a hU6-F (5'-GAGGGCCTATTTCCCATGATT-3') primer was used to confirm the identities of the DNA extracted.

DNA Extraction-Midiprep

Positive clones containing guides RBL 1-1, RBL 1-2, RBL 2-1, and 2-2 were picked from each cell line. A positive clone, 2 mL of LB media, and 2 uL ampicillin were mixed together for each cell line and incubated on a shaker (250 rpm) at 37 ^{o}C overnight. 2 mL of every mixture was then added to 48 mL of LB media each and incubated on a shaker (250 rpm) at 37 ^{o}C overnight.

The cultures were harvested by spinning on a Thermo Scientific Sorvall Lynx 4000 Centrifuge at 6000 g for 15 minutes at 4 ^{o}C before the supernatants were decanted. The pellets were resuspended in 4 mL of P1 buffer. 300 uL of P2 buffer was added to each of the solutions, which were mixed by inversion and incubated at 24 ^{o}C for 5 minutes. Next, 300 uL of P3 buffer was added and the solutions were mixed by inversion then incubation on ice for 15 minutes. The mixtures were centrifuged at 20,000 g for 30 minutes at 4 ^{o}C.

In the mean-time, QIAGEN-tips were equilibrated with 4 mL QBT buffer. The supernatant of each solution was added to a tip and entered resin by gravity flow. The tips washed with 2 rounds of 10 mL QC buffer. DNA from each solution was eluted with 5 mL QF buffer into clean vessels. This DNA was precipitated by adding 3.5 mL isopropanol and mixing. The mixtures were centrifuged at 20,000 g for 30 minutes at 4 ^{o}C before their supernatants were decanted. The DNA pellets were washed with 5 mL 70% ethanol and centrifuged at 20,000 g for 10 minutes before their supernatants were decanted. The final pellets were air-dried for 10 minutes then dissolved in 100 uL of endotoxin-free TE.

Lentivirus Packaging and Transfection

When a substantial amount of DNA was extracted, a lenti virus was prepared and used to infect cells. 24 hours before transfection, $5x10^{6}$ 293T cells were plated with 8 mL of complete growth medium on four different 10 cm plates. The plates were incubated at 37 ^{o}C, 5% CO_2 overnight.

Once the 293T cultures were 80-90% confluent, 7 ug of DNA was diluted with water to final volume of 600 uL and mixed thoroughly by vortexing.

Plasmid	DNA Concentration (ng/uL)	DNA amount (ug)	Plasmid Volume (uL)	H_2O Volume (uL)
RBL 1-1	639.964	7	10.9	589.1
RBL 1-2	917.749	7	7.6	592.4
RBL 2-1	1160.25	7	6.0	594
RBL 2-2	992.074	7	7.1	592.9

For each of these plasmids, 600 uL of diluted DNA was added to a tube of Lenti-X Packaging Single Shots (X-fect Transfection Reagent, pVSVG plasmid, pDR9-81 plasmid) then vortexed for 20 seconds. The samples were incubated for 10 minutes at room temperature and centrifuged for 2 seconds. The solutions were then added to cell plates dropwise. These plates were incubated at 37 ^{o}C, 5% CO_2 for 24 hours. 6 mL of complete growth medium was added to the plates before incubation at 37 ^{o}C, 5% CO_2 for 48 hours. Lentiviral supernatants were collected at 48 hours and 72 hours after transfection.

The viral supernatants were harvested with 0.45 um filter. 0.4 volumes of PEG/NaCl solution (30% PEG -8000/1.6MNaCl, 300 gm PEG, 93.5 gm NaCl per liter) was added to each mixture and they were incubated at -20 ^{o}C overnight. The mixtures were centrifuged at 2000 g for 30 minutes at 4 ^{o}C before supernatants were decanted. The pellets were resuspended in 2 mL full growth media (DMEM, 5% FBS, 1X penicillin/streptomycin) and stored at -80 ^{o}C in 1 mL aliquots.

Viral Infection of cells

24 hours before infection, RPE cells were plated in 12 wells with 1 mL of complete growth medium in each. 12 well-plate incubated at 37 ^{o}C, 5% CO_2 overnight. 8 mL of complete

growth medium was mixed with 8 uL of polybrene, a transfection reagent that neutralizes the charge repulsion between virions and a host cell's surface to increase the efficiency of transduction. The previous media was removed from 6 of the wells and replaced with 1 mL of the complete growth medium and polybrene mixture. 40 uL of RBL 1-1 virus was added to well 1, 40 uL of RBL 1-2 virus was added to well 2, 40 uL of RBL 2-1 virus was added to well 3, and 40 uL of RBL 2-2 virus was added to well 4. This plate was centrifuged at 2500 rpm for 1 hour at 24 ^{o}C then incubated at 37 ^{o}C, 5% CO_2 overnight.

8 mL of complete growth medium was mixed with 16 uL of blastocyrin (BLAST). The previous media was removed from 6 of the wells and replaced with 1 mL of the complete growth medium and BLAST mixture. The media from these 6 wells were moved to a new 6 well plate which was incubated at 37 ^{o}C, 5% CO_2 overnight.

Protein Quantification

The antibodies that would be used to confirm the RBL 1 and 2 KOs had to be tested in a Western blot. A plate was prepared with nine wells to quantify the amount of protein present in each of the antibodies.

Solutions	Well 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9
BCA	20 uL	10 uL	5 uL	2.5 uL	1.25 uL	0	0	0	0

PBS	0	10 uL	15 uL	17.5 uL	18.75 uL	18 uL	18 ul	18 uL	18 uL
B-Actin	0	0	0	0	0	0	2 uL	0	0
P107	0	0	0	0	0	0	0	2 uL	0
P130	0	0	0	0	0	0	0	0	2 uL

294 uL of A buffer and 6 uL of B buffer were added to each well. The plate was put on a heat block at 95 ^{o}C for 5 minutes. A BCA assay was read at 562 nm.

Western Blot Analysis of Antibodies

Two 6% resolving gels were made by mixing 5.3 mLH_2O, 2.0 mL 30% acrylamide, 2.5 mL 1.5 M Tris, 100 uL 10% SDS, 100 uL 10% APS, and 8 uL TEMED. 1 mL Isopropanol was added to each gel to quench the reaction. A 4% stacking gel was made by mixing 3.05 mL H_2O, 1.25 mL protogel stacking buffer, 650 uL protogel, 25 uL 10% APS, and 5 uL TEMED. 1 mL Isopropanol was added to quench the reaction. 1X Tris Buffered Saline with Tween 20 (TBST) was made with 200 mL 10X Tris Buffered Saline (TBS), 1790 mL mL ddH_2O, and 10 mL Tween 20.

30 ug of each protein (RPE1 and RPE2) was mixed with 15-20 uL radioimmunoprecipitation assay (RIPA) buffer and 10 uL 3x loading buffer (LB) to get volume up to 30 uL. These samples were boiled for 4 minutes, placed on ice for 2 minutes, spun down,

and loaded onto wells 1 and 2 of the gel. A molecular weight ladder (5 uL of BIO-RAD color marker, 5 uL of Invitrogen magic marker, 10 uL of 3x LB, 10 uL RIPA buffer) was added to well 3 of the gel. A BIO-RAD Power Pac was attached to the BIO-RAD Wide Mini-Sub Cell GT holding the gel and run at 120 V then ramped up to 150 V for 2 hours.

A membrane was activated by putting it in about 5 mL TransBlot Turbo 1X buffer (200 mL TransBlot Turbo 5X buffer, 200 mL 100% ethanol, and 600 mL ddH_2O). The gel was placed on top of the activated membrane in a cassette of the TransBlot Turbo Transfer pack. The cassette was locked and placed in the TransBlot machine to run the transfer. Once the transfer was complete, membrane was placed in blocking buffer (5 g milk powder, 50 mL 1X TBST) and incubated on a shaker (250 rpm) at 37 ^oC overnight.

The membrane was placed in 5 mL of wash buffer (0.5 g milk powder, 100 mL 1X TBST) for 15 minutes before the buffer was removed. 5 mL of wash buffer was added before incubation on a shaker (250 rpm) for 5 minutes then the buffer was removed, a process that was repeated. The membrane was then separated based on the molecular ladder into p107, p130, and β-actin sections. All sections were placed in separate sections in 4-5 mL of their respective primary antibodies diluted with wash buffer and incubated on a shaker (250 rpm) for 30 minutes.

Primary Dilutions	Volume Antibody (uL)	Volume Wash Buffer (uL)
B-Actin	1	5000
P107	20	4000
P130	20	4000

First, 20 mL of wash buffer was added to membrane and quickly removed. High salt buffer (0.45 g of milk powder, 90 mL of TBST/0.5 NaCl solution) was then added to remove any

primary antibodies that did not bind to protein from the membrane. The membrane was placed in 5 mL of high salt buffer and incubated on a shaker (250 rpm) for 15 minutes before the buffer was removed. 5 mL of wash buffer was added before incubation on a shaker (250 rpm) for 5 minutes then the buffer was removed, a process that was repeated. The membrane was then placed in 4-5 mL of secondary antibodies diluted with wash buffer and incubated on a shaker (250 rpm) for 30 minutes.

Secondary Dilutions	Volume Antibody (uL)	Volume Wash Buffer (uL)
B-Actin (mouse, Sigma Aldrich)	1	4000
P107 (rabbit, GE Healthcare, 931V)	8	4000
P130 (rabbit, GE Healthcare, 934V)	8	4000

20 mL of wash buffer was added to the membrane and quickly removed. The membrane was then placed in 5 mL of high salt buffer and incubated on a shaker (250 rpm) for 15 minutes before the buffer was removed. 5 mL of wash buffer was added before incubation on a shaker (250 rpm) for 5 minutes then the buffer was removed, a process that was repeated twice. 1 mL of ECL buffer was added to membrane and then put in the Li-Cor machine to visualize bands.

Western Blot Analysis of KOs

After the antibodies were shown to be of good quality, the CRISPR-KO cells were tested with a Western blot. First, media was removed from each of the cell lines. The cells were washed in 1X PBS and placed on ice. The PBS was removed, and each cell line was mixed with 100 uL 4% SDS before being placed on ice. Samples were sonicated with several pulses on setting 3

then incubated on ice for 30 minutes. The samples were then centrifuged at 2000 g for 10 minutes at 4 ^{o}C. 2 uL of each sample was taken for protein quantification and the rest of the samples were stored at -80 ^{o}C.

A plate was prepared with ten wells to quantify the amount of protein in each of the KOs.

Solutions	Well 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 10
BCA	20 uL	10 uL	5 uL	2.5 uL	1.25 uL	0	0	0	0	0
PBS	0	10 uL	15 uL	17.5 uL	18.75 uL	18 uL	18 ul	18 uL	18 uL	18 uL
RBL 1-1	0	0	0	0	0	0	2 uL	0	0	0
RBL 1-2	0	0	0	0	0	0	0	2 uL	0	0
RBL 2-1	0	0	0	0	0	0	0	0	2 uL	0
RBL 2-2	0	0	0	0	0	0	0	0	0	2 uL

294 uL of A buffer and 6 uL of B buffer were added to each well. The plate was put on a heat block at 95 ^{o}C for 5 minutes. A BCA assay was read at 562 nm. Samples stored at -80 ^{o}C

A 4–20% Mini-PROTEAN TGX™ Precast 15-well (15 uL) Protein Gel was loaded with a blank (70 mL RIPA buffer, 35 mL 3x LB buffer) in lanes 1 and 15, a color marker (15 uL of

BIO-RAD color marker, 30 uL of blank) in lanes 2, 8, and 14, a magic marker (10 uL of Invitrogen magic marker, 20 uL of blank) in lanes 3 and 13, and samples in lanes 4-7 and 9-12. The samples were made up of:

Well #	Sample	ug/uL	V protein for 20 ug/well (uL)	V RIPA buffer (uL)	V 3x LB (uL)
4 & 9	Fus 1	3.51	11.40	8.6	10
5 & 10	Fus 2	3.94	10.15	9.85	10
6	RBL 1-1	2.82	7.09	2.91	5
7	RBL 1-2	2.8	7.14	2.86	5
11	RBL 2-1	2.82	7.09	2.91	5
12	RBL 2-2	2.8	7.14	2.86	5

The samples were boiled for 4 minutes, placed on ice for 2 minutes, spun down, and loaded onto gel. A BIO-RAD Power Pac was attached to the BIO-RAD Wide Mini-Sub Cell GT holding the gel and run at 130 V then ramped up to 150 V for 2 hours.

A membrane was activated by putting it in about 5 mL TransBlot Turbo 1X buffer. The gel was placed on top of the activated membrane in a cassette of the TransBlot Turbo Transfer pack. The cassette was locked and placed in the TransBlot machine to run the transfer. Once the transfer was complete, membrane was placed in blocking buffer and incubated on a shaker (250 rpm) at 37 ^{o}C overnight.

The membrane was placed in 5 mL of wash buffer for 15 minutes before the buffer was removed. 5 mL of wash buffer was added before incubation on a shaker (250 rpm) for 5 minutes then the buffer was removed, a process that was repeated. The membrane was then separated

based on the molecular ladder [cuts at 250, 75, and 25 kB then on central CM for top section] into p107, p130, and β-actin sections. All sections were placed in separate sections in 2-4 mL [determined by the size of the partial membrane in the section] of their respective primary antibodies diluted with wash buffer and incubated on a shaker (250 rpm) for 90 minutes.

Primary Dilutions	Volume Antibody (uL)	Volume Wash Buffer (uL)
B-Actin	0.8	4000
P107	10	2000
P130	10	2000

First, 7-14 mL of wash buffer was added to each section of the membrane and quickly removed. Each section of the membrane was then placed in 3.5-7 mL of high salt buffer and incubated on a shaker for 15 minutes before the buffer was removed. 2-4 mL of wash buffer was added to each before incubation on a shaker for 5 minutes then the buffer was removed, a process that was repeated. Each section of the membrane was then placed in 2-4 mL of secondary antibodies diluted with wash buffer and incubated on a shaker for 45 minutes.

Secondary Dilutions	Volume Antibody (uL)	Volume Wash Buffer (uL)
B-Actin (mouse, Sigma Aldrich)	1	4000
P107 (rabbit, GE Healthcare UK, 931V)	4	2000
P130 (rabbit, GE Healthcare UK, NA 934V)	4	2000

7-14 mL of wash buffer was added to each section of the membrane and quickly removed. Each section of the membrane was then placed in 3.5-7 mL of high salt buffer and incubated on a shaker for 15 minutes before the buffer was removed. 2-4 mL of wash buffer was added to each well before incubation on a shaker for 5 minutes then the buffer was removed, a process that was repeated twice. 1 mL of ECL buffer was added to membrane and then put in the Li-Cor machine to visualize bands.

Results

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The RNA, which had their OD readings done on the NanoDrop spectrophotometer, all had 260/280 ratios between 1.8 and 2.1, showing that they had high quality. The purity and quality of this RNA was further confirmed by the fact that discrete bands had shown at 28S, 18S, and 5S -matching the RNA reference ladder- when visualized in the Agilent Bioanalyzer.

The results from the first round of qRT-PCR informed the rest of my project. Consistent melt curves [Figure 8] for all samples established that each contained quality primers.

The amplification plots [Figure 9] from both qRT-PCR rounds show that genes start amplifying between cycle 20 and 24 based on the AT content of their primer. The more AT-rich a primer is, the quicker amplification starts as there is a parallel trend with melting temperature.

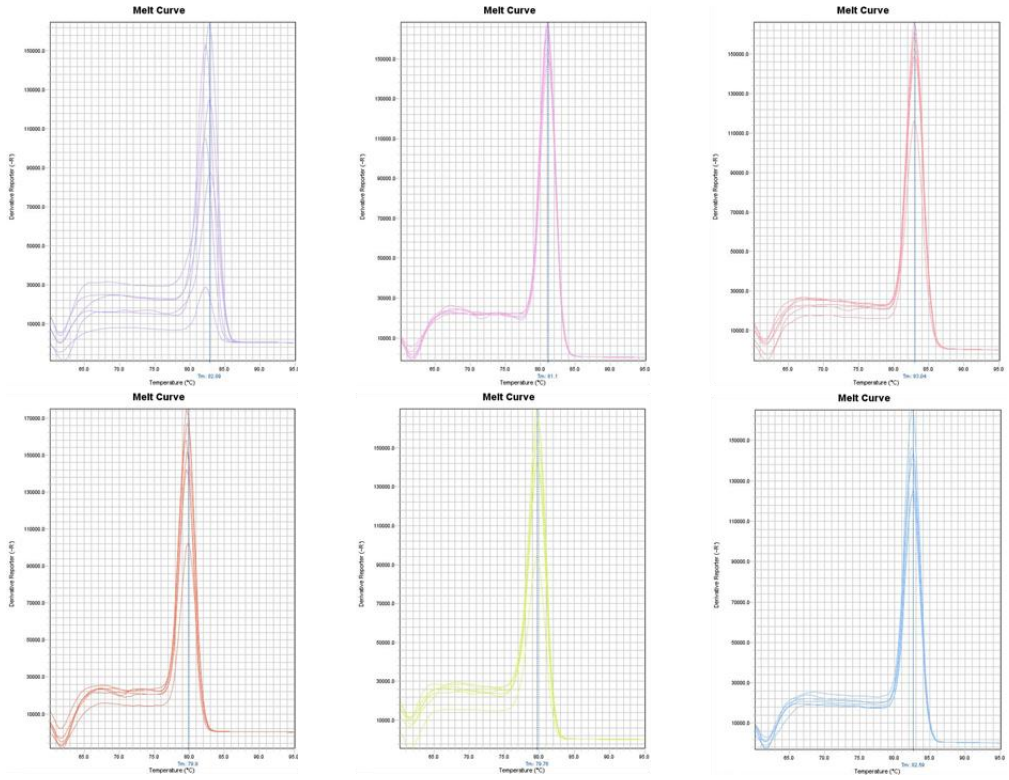

Figure 8: Melt Curves of 6 primers

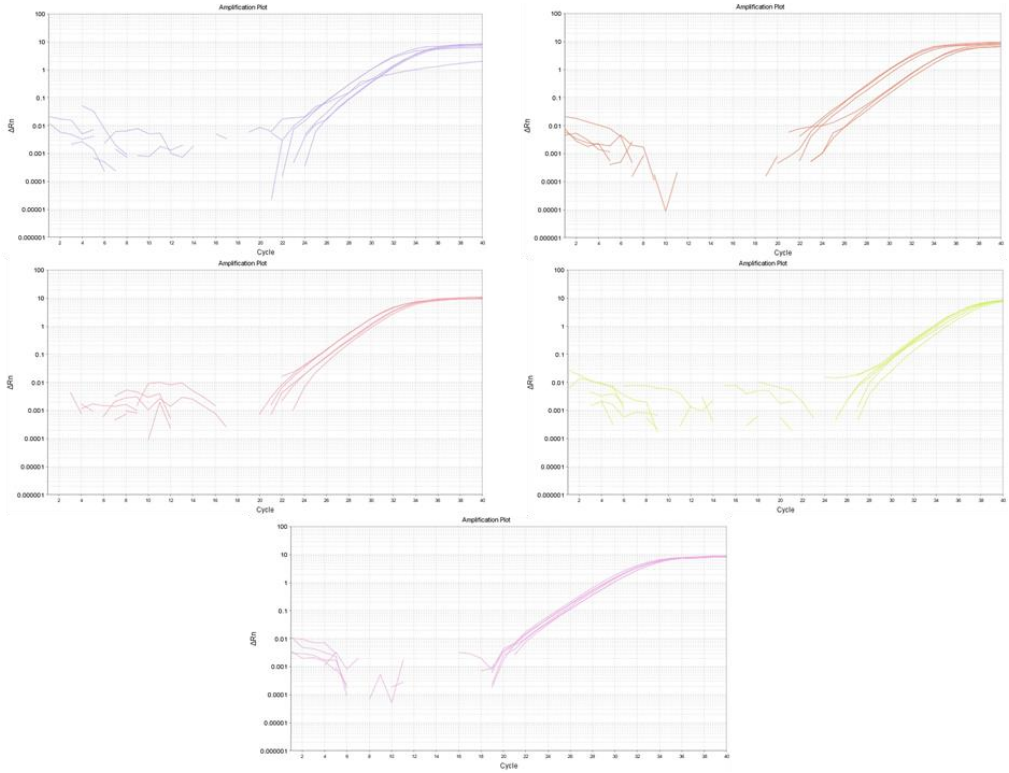

Figure 9: Amplification Plots of 6 primers

In cell lines containing NF-κB, genes of interest had considerably greater expression than those that lacked NF-κB.

After the comparison test was performed, 16 of the 35 target genes showed differential expression ($p<0.05$), were deemed genes of interest moving forward and thus grouped together. After the student T-test was performed, 12 of the 35 target genes showed differential expression ($p<0.05$), were deemed genes of interest moving forward and again, and were grouped together.

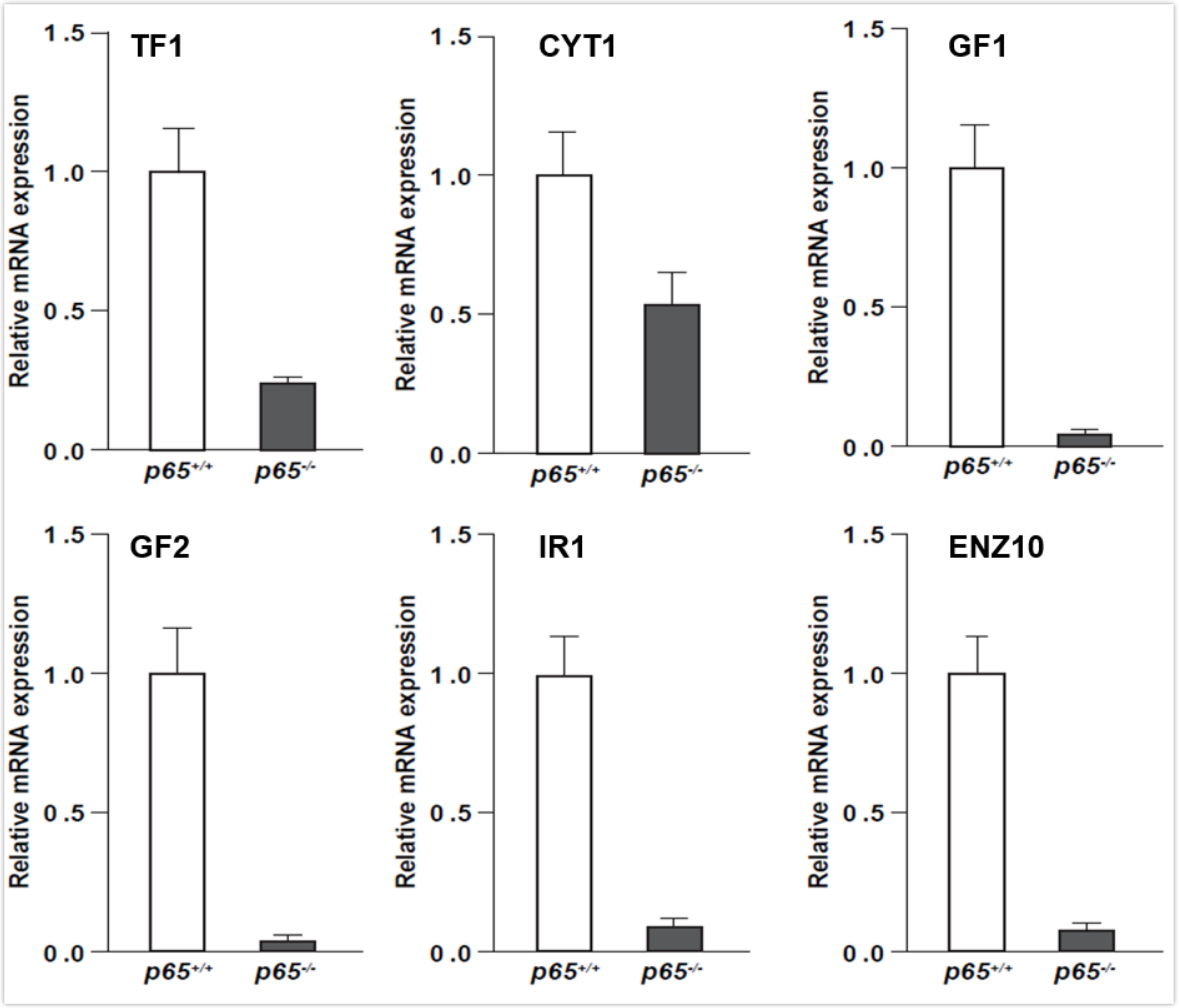

Figure 10: The differential ($p < 0.05$) expression between 6 of the 12 genes of interest. 5 of the 6 shown, CYT1 being the exclusion, have a p value <0.01.

Six of these 12 differentially expressed genes had functions that seemed especially important in a cancer-causing pathway [Figure 10].

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DNA extraction with a mini prep kit was successful, having decent yields of DNA (ng/uL) and read quality (260/280 ratio).

Name	260 Raw	280 Raw	320 Raw	260	280	260/280	ng/µL
RBL 1-1	0.197	0.16	0.104	0.087	0.053	1.659	87.169
RBL 1-2	0.086	0.071	0.053	0.028	0.015	1.866	28.089
RBL 2-1	0.358	0.221	0.055	0.296	0.161	1.832	295.743
RBL 2-2	0.1	0.079	0.054	0.041	0.022	1.856	41.361

Sanger sequencing from DNA extracted in the mini prep protocol confirmed the identities of each sample. The region that is not highlighted represents the plasmid, the green highlighted region is the consensus leader region of the guide, and the yellow highlighted region represents the specific DNA sequence of the guide.

DNA identity	Sanger sequence
RBL 1-1	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGTGGTGATCCGCTCAGAAGAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
RBL 1-2	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGCTACAGTTCTCCTACCGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
RBL 2-1	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCCGCCTCAACATGGACGAGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
RBL 2-2	CTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGGCGACTGGTCACCTCCCGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG

The western blot of the antibodies [Figure 11] that would test KOs showed that they would all have utility in upcoming tests of RBL 1 and 2 KOs.

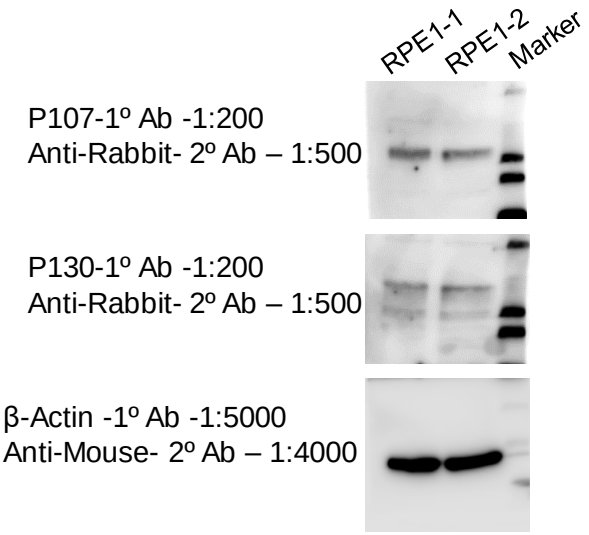

Figure 11: The validation of the primary and secondary antibodies for RBL 1 & RBL 2

DNA extraction with a midi prep kit was successful, having decent yields of DNA (ng/uL) and read quality (260/280 ratio).

Name	260 Raw	280 Raw	320 Raw	260	280	260/280	ng/µL
RBL 1-1	0.713	0.413	0.059	0.64	0.346	1.848	639.964
RBL 1-2	0.999	0.574	0.06	0.918	0.503	1.826	917.749
RBL 2-1	1.23	0.692	0.054	1.16	0.63	1.843	1160.25
RBL 2-2	1.069	0.607	0.061	0.992	0.538	1.845	992.074

293T cells [Figure 12], visualized by an EVOS™ XL Core Imaging System, were used in conjunction with lentivirus because they allow for mass transfection.

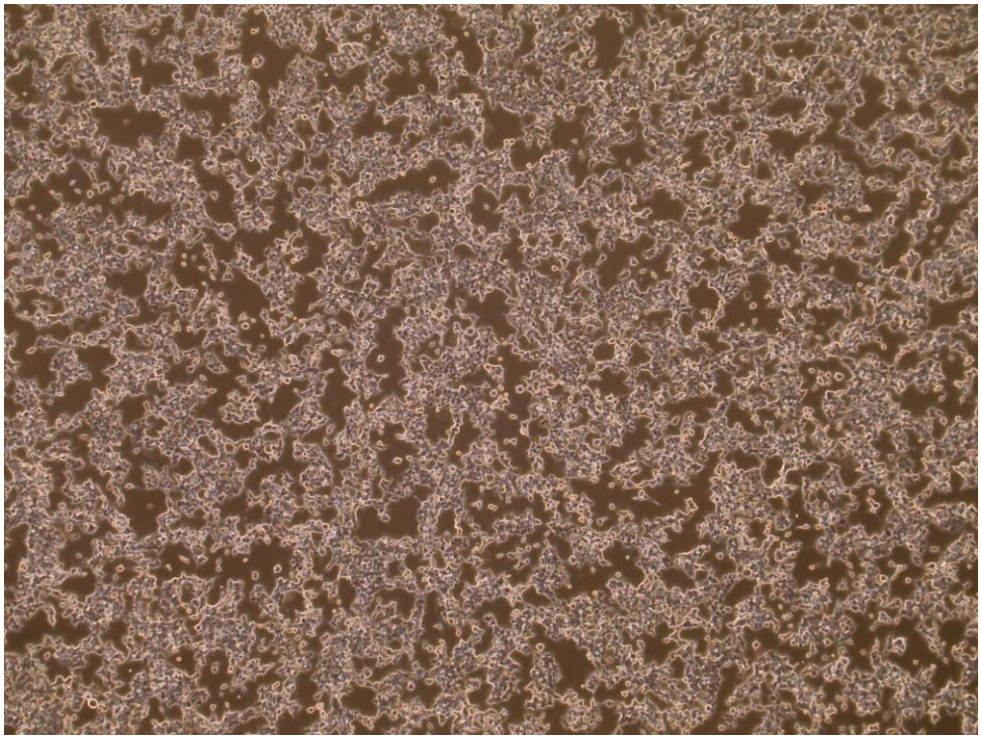

Figure 12: 293T Cells used during transfection with lentivirus

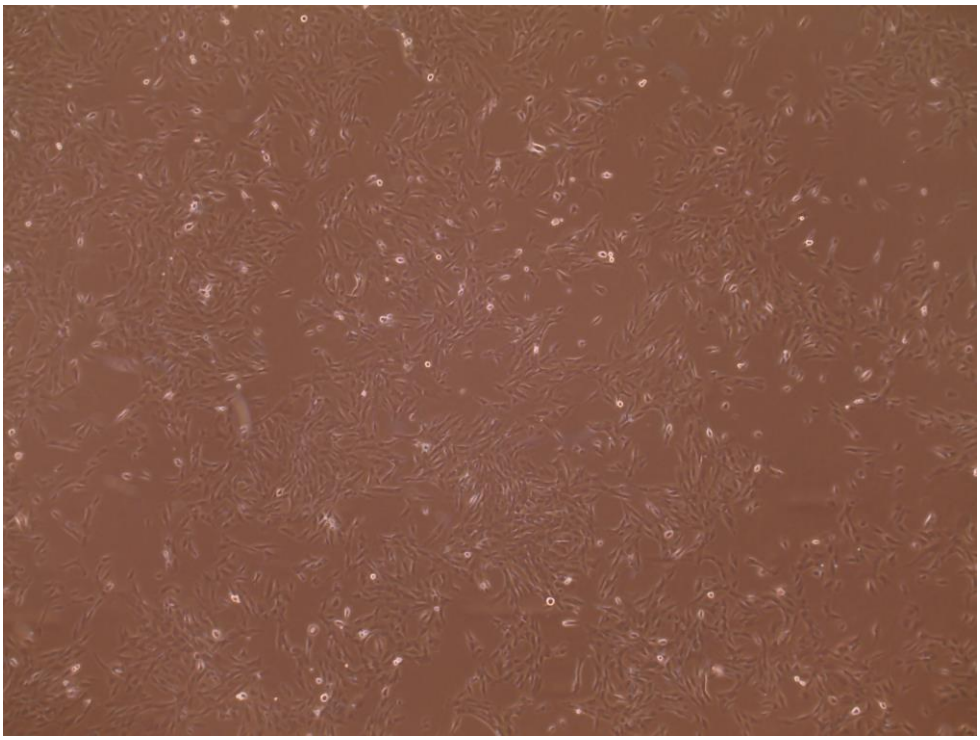

Figure 13: RPE cells that were infected with viral supernatant later used during selection of CRISPR-KO cell lines

RPE cells [Figure 13], visualized by an EVOS™ XL Core Imaging System, were used

because they are one of the only cell lines with an intact RB/E2F pathway. This allowed the Western Blot to show the true impact of RBL 1 & RBL 2 KOs.

The Western Blot of the CRISPR-KOs [Figure 14] compared either the expression of p107 (for RBL 1 KOs) or p130 (for RBL 2 KOs) among the KOs and two negative CRISPR control cell lines (Fus 1 and Fus 2) with intact RBL 1 and RBL 2 genes. It standardized all the cell lines against a loading control (β-actin). All KOs had differential protein expression from the Fus cell lines, with RBL 1-1 appearing to be a successful KO of RBL 1 in addition to both RBL 2-1 and RBL 2-2 performing as complete RBL 2 KOs.

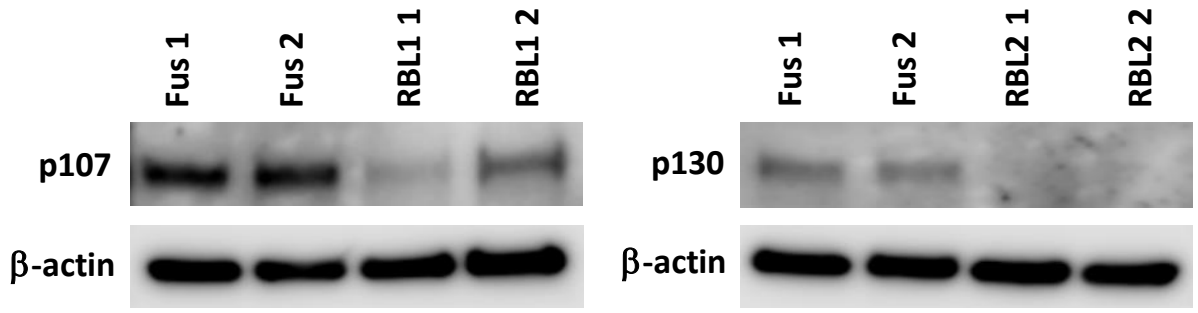

Figure 14: Western Blot was run comparing the expression of β-actin and either p107 or p130 among the CRISPR KOs and control cell lines.

Conclusions

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Primers were designed that were able to confirm differentially regulated genes. With successful amplifications in the first round of PCR analysis, I identified 16 differentially regulated genes that had not been previously reported as being regulated by NF-κB. The second round of PCR analysis revealed that 12 genes of interest had significant differential regulation in NF-κB wild-type and mutant cell lines. In conclusion, I was able to narrow 35 genes encoding secreted factors to 12 possible NF-κB regulated genes.

Future experiments will be performed to further analyze these genes to better understand how NF-κB can contribute to cancer development through cell survival, cell growth or immune suppression. If the way that the NF-κB pathway specifically helps cancer cells evade the immune system becomes clearer, a novel cancer therapy can be created to counter this mechanism and inhibit the entire pathway. A therapy of this nature could affect rapidly progressing cancers since constitutively active cancer cells appear to have a "NF-κB addiction" (Chaturvedi M.M., 2011).

The first step to understand how these genes of interest can contribute to a cancer-causing pathway will be to test them in a cancer cell line where NF-κB is constitutively activated. Differentially expressed genes will be analyzed with RNA prepared from the cell lines of pancreatic cancer-afflicted mice with successful primers. The experiment will be run in triplicate, and t tests will be utilized to further confirm the statistical difference between $p65^{+/+}$*Ras* and $p65^{-/-}$*Ras* lines derived from *KRasG12D;* $p53^{-/-}$*; Pdx-Cre*$^{+/-}$ (F) cell lines. After this, NF-κB binding sites will be investigated for each of the genes of interest on the rVista database to view how gene may be regulated within the pathway. The electrophoretic mobility shift assay

(EMSA), chromatin immunoprecipitation (ChIP) and luciferase assay method can then be utilized to confirm NF-κB regulation of these genes. Additionally, an exploration will be made on the National Institute of Health's The Cancer Genome Atlas (TCGA) to determine how the expression of genes of interest in different cancer patients predicts patient outcomes (survival). This will be necessary to see how these genes' functions may change in different cancerous environments. Lastly, a knock-down or knock-out of these genes will be made in human cells to see if either can affect tumor development in tumor models. With all this information, one would be able to extrapolate signal transduction pathways to further understand how any certain gene is contributing to a cancerous environment.

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Three cell lines (RBL 1-1, RBL 2-1, RBL 2-2) were created that performed as phenotypic KOs of the gene they were specified for with CRISPR-Cas9 gene editing. This is important as a clone can be selected from any of these lines to eventually create *bonafide* RBL 1 or RBL 2 KO cell lineages that can be tested a multitude of times to understand the function of these genes, and their proteins, in the human body. Once the impact of these KOs is understood, they can act as a model for how p107 or p130 can act in a cancer-causing pathway as well. The fourth cell line, RBL 1-2, also serves as a partial knockdown and could conceivably contain a good enough KO clone to spawn its own RBL 1 KO lineage.

In the future, KO clones will be selected, and western blots will be run to confirm KO of RBL 1 and RBL 2. At least two clones will be cultured for RBL 1 and RBL 2 each to ensure that the changes observed are not due to off-target effects. KO cell lines will be further characterized by functional and high throughput assays to determine the function of RBL proteins.

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